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14. ABSTRACT

A polypurine tract (PPT) containing multiple GGA repeats in the HER-2/neu promoter is important to control HER-2/neu transcription. We investigated the ability of the PPT to form a G-quadruplex-related secondary structure using biochemical techniques and screened a small family of potential G-quadruplex ligands that could stabilize PPT secondary structure formation in solution. Telomestatin and a lead compound in the fluoroquinolone class stabilize the HER-2/neu PPT secondary structure in solution and reduce HER-2/neu expression in breast cancer cells. We conclude the HER-2/neu promoter can form a stable secondary structure known as a tetrad:heptad in solution. Further studies are needed to fully characterize the secondary structure and link the effects of compounds on HER-2/neu expression to their direct interaction with the HER-2/neu promoter using reporter cell lines that are currently under construction. We also identified a second potential therapeutic target in the HIF-1 alpha gene promoter capable of forming a G-quadruplex structure that can also bind to G-quadruplex ligands and serve as a basis of developing small molecule inhibitors of gene transcription for the treatment of breast cancer.

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Introduction

The central hypothesis of this project is that the formation of a tetrad:heptad variant of a G-quadruplex by the HER-2/neu promoter represses HER-2/neu transcription. The tetrad:heptad provides a target for selective drug-DNA interaction by G-quadruplex interactive compounds and inducing or stabilizing the tetrad:heptad in the HER-2/neu promoter with a G-quadruplex interactive compound will silence HER-2/neu expression.

The specific aims of this proposal are:

Specific Aim 1: Investigation of the potential biological role of a tetrad:heptad DNA structure in the HER-2/neu promoter in the regulation of HER-2/neu gene expression.

Specific Aim 2: Investigation of the ability of quadruplex selective DNA interactive compounds to target the tetrad:heptad structure in the HER-2/neu promoter *in vitro* and *in vivo*.

In order to accomplish these aims, we proposed a series of experiments to evaluate the ability of G-quadruplex interactive compounds to bind to the HER-2/neu promoter in solution and prevent HER-2/neu expression in cells. In our statement of work, we proposed to initiate these experiments in year 1 to identify lead compounds, and continue their characterization in years 2 and 3. In our statement of work, we proposed to initiate biochemical studies in year 2 to further characterize the structure formed by the HER-2/neu promoter in an attempt to understand the potential biological role of DNA secondary structure in the regulation of HER-2/neu expression. In this annual report, we describe our preliminary findings on the G-quadruplex interactive compounds that we screened for interaction with the HER-2/neu promoter and on further characterization of the DNA secondary structure formed by the polypurine tract in the HER-2/neu promoter.

Body

The HER-2/neu oncogene is frequently overexpressed in breast cancer and represents an important therapeutic target. HER-2/neu expression is frequently disproportionate to gene copy number with or without gene amplification as a result of transcriptional activation. A polypurine tract (PPT) containing multiple GGA repeats is an important promoter element in the control of HER-2/neu transcription. GGA repeats have been shown to form unusual DNA structures related to guanine (G) quadruplexes at physiological potassium concentrations. G quadruplexes are emerging as potential therapeutic targets for the treatment of cancer.

We propose that the PPT might be able to fold into an intramolecular quadruplex within cells. From previous studies it was known that insertion of GGA/TCC repeats into plasmid DNA makes that region sensitive to S1 nuclease, suggesting the formation of secondary DNA structure (*1*). Recently NMR data showed that sequences containing four consecutive GGA repeats form a very stable quadruplex variant under physiological potassium concentrations (*2;3*). This structure, called a tetrad:heptad (**Figure 1**), is composed of a typical guanine tetrad stacked on a guanine-adenine heptad containing 4 guanines and 3 adenines. The purine rich tract of the HER-2/neu promoter contains the first 11 nucleotides of $(GGA)_4$ needed to form a tetrad:heptad and quadruplex formation

could prevent the binding of essential transcriptional factors, such as Ets, thus decreasing HER-2/neu transcription. Quadruplex formation in the HER-2/neu promoter might also be enhanced by the addition of DNA interactive compounds that selectively bind and stabilize quadruplex formation (*4*).

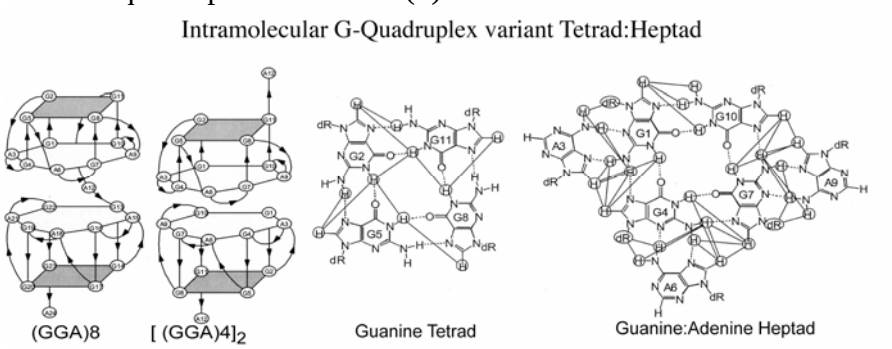

Figure 1. Tetrad:heptad DNA structures formed by oligos with four or eight GGA repeats.

In our annual report last year, we described the following accomplishments on this project, which had been achieved through the end of the first grant year:

1. *Screening compounds with Taq Pol arrest assay identified several potential HER-2/neu transcriptional inhibitors.* To investigate if G-quadruplex interactive compounds are able to induce the secondary structure in the HER2/neu PPT, we used a modification of a published technique, the DNA polymerase (Taq Pol) arrest assay (*5;6*). We evaluated two commercially available compounds, the cationic porphyrins TmPyP2 and TmPyP4 (*7-10*), telomestatin, a natural product isolated from *Streptomyces anulatus* (*7-10*), and twelve proprietary compounds based on a common fluoroquinolone pharmacophore (*11;12*) provided to us by Cylene Pharmaceuticals (San Diego, CA). Telomestatin and 4 of the 12 compounds from Cylene stabilized the secondary structure within the HER-2/neu PPT at sub-micromolar concentrations.
2. *Demonstration that compounds identified by this assay can suppress HER-2/neu expression in cells.* Telomestatin modestly reduced HER-2/neu expression at non-toxic doses in the colon cancer cell line COLO-205 (top panel) that aberrantly co-overexpresses HER-2/neu and c-MYB (co-expression of c-MYB is of interest because c-MYB contains a highly homologous GGA repeat sequence in its 5'untranslated region). The porphyrins (TmPyP4 and P2) had no effect on HER-2 expression as predicted by the TaqPol assay.
3. *Identification of a lead compound, CX1398 for further evaluation against various breast cancer cell lines.* We also investigated the effects of a second generation of Cylene compounds on HER-2/neu expression in the breast cancer cell line MDA-MB-175-VII (bottom panel). This cell line is a good model for transcriptional overexpression of HER-2/neu, since it overexpresses HER-2/neu mRNA 10-fold from a single copy of the HER-2/neu gene. CX1398 had a significant effect on HER-2/neu transcription, decreasing expression by 70%.
4. *Biochemical characterization of the HER-2/neu promoter in solution.*

A. *Circular dichroism studies demonstrate identity with the NMR tetrad:heptad structure.* The CD spectra of oligos representing the HER-2/neu promoter were identical to the spectrum reported for the $(GGA)_4$ oligo by Matsugami *et al* in their initial report of the tetrad:heptad structure (*2*). These observations imply that the HER2/neu promoter polypurine tract contains a sequence capable of tetrad:heptad formation.

B. *EMSAs and footprinting studies demonstrate competing structures in solution.* We found that longer oligos have complex electrophoretic mobility and chemical cleavage protection patterns that are probably derived from competing intermolecular structures in solution. For these reasons, it is difficult to fully characterize the structure formed by the HER-2/neu promoter with biochemical techniques and firmly conclude that the GGA repeats form a tetrad:heptad structure.

At the end of the first year of this award, we concluded: "the GGA repeats in the HER-2/neu promoter can probably form a stable secondary structure known as a tetrad:heptad, although the biochemical characterization of the secondary structure formed by the full length of the HER-2/neu PPT has proven difficult because of competing secondary structures by these G-rich oligos." For the second year of this award, we proposed to begin the challenging task of proving that the G-quadruplex structure is involved in regulating HER-2/neu promoter function in breast cancer cells by using HER-2/neu promoter-luciferase reporter constructs, and comparing the promoter activity in wild-type versus G-quadruplex mutant promoters, particularly after treatment with G-quadruplex interactive compounds, such as our lead CX1398 compound, on HER-2/neu driven luciferase expression.

HER-2/neu promoter-luciferase reporter gene constructs.

For these studies, we began with two plasmids bearing 410 bp of the HER-2/neu promoter (from the initiation codon, +1, through -410, and numbered from the translation start site), upstream of the luciferase gene in the pGL3 basic vector (Promega). These plasmids contain the minimal essential HER-2/neu promoter. The construct with the native HER-2/neu promoter is named pGL3/HNP410. A promoter with 5 point mutations (underlined) in the tetrad:heptad forming region (bold) was created by site-directed mutagenesis and named pGL3/HNP410(Δ-225):

Wild type (GGA repeat region): TCACAGGAGAAGGAGGAGGT**GGAGGAGGAGG**GCTGC
Δ-225 mutation: TCACAGGAGAAGGAGGAGGT**GGAGGCATGCG**GCTGC

In order to derive stable transfectants, we transferred the native or mutant HER-2/neu promoter fragment from pGL3/HNP410 into the pGL4.20 plasmid (Promega), which contains a mammalian selection marker for puromycin (as well as some improvements in the luciferase reporter system), resulting in pGL4.20/HNP386 and pGL4.20/HNP386(Δ-225). These plasmids are intended for transfection and stable selection in ZR-75-1 cells, a breast cancer cell line that is ideal for evaluating the potential role of the GGA repeats in the regulation of the HER-2/neu promoter, because these cells overexpress HER-2/neu by

about 5-10 fold from a single copy of the HER-2/neu gene. Thus, in ZR-75-1 cells, HER-2/neu overexpression is caused by increased transcription from the HER-2/neu promoter.

In order to create completely isogenic reporter cell lines bearing a single, integrated HER-2/neu promoter-luciferase reporter gene, we transferred the native or mutant HER-2/neu promoters together with the entire luciferase coding sequence from pGL4.20/HNP386 into the pcDNA5/FRT plasmid (Invitrogen), the latter modified by entirely removing the CMV promoter. The resulting vectors are named pHNP386-luc/FRT and pHNP386(Δ-225)-luc/FRT. These vectors are useful for creating stable, integrated genes in host cells bearing a flp recombinase target sequence (FRT) using the flp recombinase enzyme. A summary of the reporter constructs created for this project is presented in Table 1.

Table 1. HER-2/neu Promoter-Luciferase Constructs

Name	*Vector*	*Description/Use*
pGL3/HNP410 ***pGL3/HNP410(Δ-225)***	pGL3-basic (Promega)	Minimal HER-2/neu 410bp promoter drives luciferase expression for transient transfections and as a source of promoter DNA for cell-free studies. Mutant plasmid bears 5 point mutations in the GGA repeats.
pGL4.20/HNP386 ***pGL4.20/HNP386(Δ-225)***	pGL4.20 (Promega)	Vector engineered to express a mammalian selection marker, puromycin resistance, and to reduce background luciferase expression due to cryptic transcription factor binding sites.
pHNP386-luc/FRT ***pHNP386(Δ-225)-luc/FRT***	pcDNA5/FRT (Invitrogen)	Vector bearing a FRT and hygromycin resistance selection marker, used to create stable, single-integrants of wild-type and mutant HNP386-luciferase gene construct in host cells that bear a FRT.

S1 nuclease mapping of the HER-2/neu promoter in a supercoiled plasmid.
We hypothesized that supercoiling would induce G-quadruplex formation in the G-rich region of the HER2/neu promoter in the presence of KCl creating an unpaired pyrimidine strand sensitive to S1 cleavage. S1 nuclease probing of the plasmid pGL3/HNP410 was used to determine if the polypurine tract of the HER2/neu promoter can adopt an alternate DNA conformation due to supercoiling. Using similar techniques, Scott *et al.*, had previously reported that the HER2/neu promoter can form Hr-DNA, an intramolecular triplex, under supercoiling pressure, but these studies were done in the presence of a slightly superphysiologic concentration (4 mM) of $MgCl_2$ and in the absence of KCl (*13*). Plasmid DNA was subjected to S1 digestion and detection by Southern blot using a probe that binds downstream of the polypurine tract (**Figure 1**). This probe is

complimentary to the bottom strand of the HER2/neu promoter. Supercoiled plasmid treated with S1 nuclease and then with NcoI (lane 6) shows several supercoiling dependent S1 sensitivity bands not seen in linearized plasmid after S1 digestion (lane 5), but the location of these S1 sensitive sites could not be determined, and cleavage on the pyrimidine-rich strand in the PPT region of a 200 bp S1-NcoI fragment was not observed.

In a very novel application of technology to determine secondary DNA structure, we used reagents from our projects to develop PNAs as anti-gene inhibitors of HER-2/neu expression to probe for the intrinsic DNA structure of the HER-2/neu promoter in supercoiled plasmids. We hypothesized that bis-PNAs targeting the G-quadruplex forming region of the HER-2/neu promoter require B-DNA for binding and thus would be inhibited from binding in the presence of an intrinsic secondary DNA structure, such as a heptad:tetrad. Because bis-PNAs create an unpaired displacement loop in the pyrimidine-rich strand of the target sequence, this displacement loop can be detected by very similar S1 digestion patterns as expected for G-quadruplex formation, and were thus also useful as a "positive control" for the formation of a secondary DNA structure creating an unpaired pyrimidine strand in the GGA repeat region. We observed that a bis-PNA targeting the GGA repeat regions of the HER-2/neu promoter created a displacement loop, shown as a 200 bp S1-NcoI fragment on the Southern blot. These data show that binding created an S1 hypersensitivity site ~200 bp upstream of the NcoI site in the pyrimidine strand of the HER2/neu PPT, and imply that the intrinsic structure of the GGA repeats in a supercoiled plasmid is double-stranded, B-DNA.

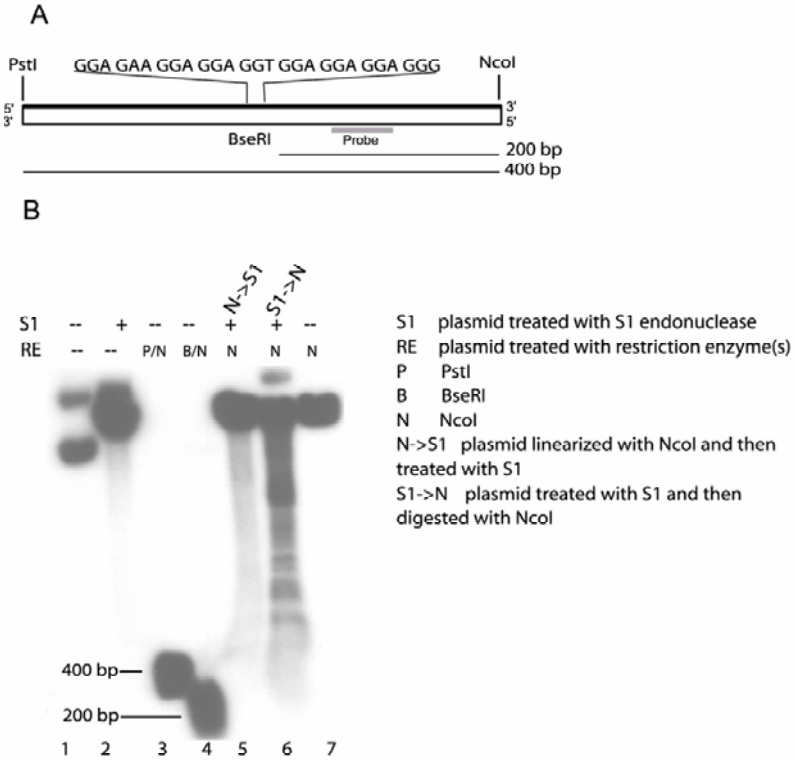

Figure 1. S1 nuclease mapping of HER2/neu promoter in supercoiled plasmid construct pGL3/HNP410. (A) Schematic representation of the HER2/neu promoter showing the location of the polypurine tract, probe, and restriction sites. The binding site of ProbePy

complimentary to the bottom (pyrimidine) strand is indicated by the gray bar. (B) S1 nuclease sensitivity assay of the HER2/neu promoter in linear and supercoiled plasmids. S1 treated (+) supercoiled plasmids or untreated plasmids (--) were linearized with NcoI (N). Plasmid linearized with NcoI was treated with S1 (N->S1) to demonstrate the effect of supercoiling on S1 endonuclease sensitivity. Double digestion with PstI/NcoI (P/N) and BseRI/NcoI (B/N) was done to demonstrate the positions of 400 and 200 bp bands representing the HER-2/neu promoter.

In summary, we did not observe a potassium induced S1 hypersensitivity site in the GGA repeat region, and the GGA repeats provided an excellent binding site for a bis-PNA in supercoiled plasmid DNA. We conclude that the intrinsic (native) structure of the GGA repeats in a supercoiled plasmid bearing the HER-2/neu promoter is B-DNA. As such, we surmise that intracellular forces in addition to supercoiling and/or G-quadruplex interactive drug binding are necessary to induce secondary structure formation in the GGA repeat region of the HER-2/neu promoter.

Transient Transfection of Native versus Quadruplex Mutant HER-2/neu Promoters

We have demonstrated that the GGA repeat region in the HER-2/neu promoter is an important element in the transcriptional control of HER-2/neu gene expression. We compared the expression of the pGL4.20/HNP386 (wild-type) HER-2/neu promoter with the pGL4.20/HNP386(Δ-225) (GGA mutant) plasmids in transient transfection assays in ZR-75-1 breast cancer cells. HNP reporter plasmids were co-transfected with a renilla luciferase control, and dual luciferase assays were performed. The data were derived from the ratio of the average firefly luciferase activity to renilla luciferase activity, and then the expression from the mutant plasmid was represented as a percentage of the wild-type expression (**Figure 2**).

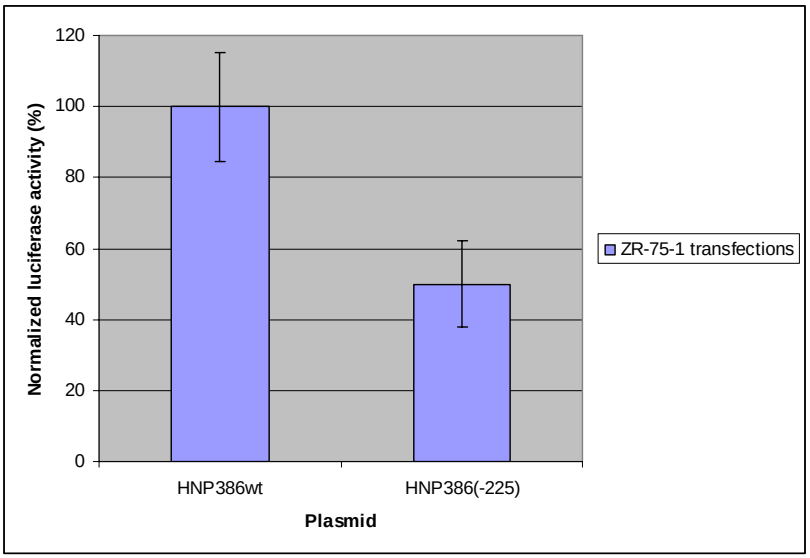

Figure 2. Transient transfection of native versus GGA mutant HER-2/neu reporter plasmids into ZR-75-1 breast cancer cells.

These data demonstrated a 50% reduction in HER-2/neu transcription by perturbing the GGA repeat region (**Figure 2**). Most likely, the reduction in HER-2/neu transcription derives from perturbing a critical transcription factor binding site in the GGA repeat region, and suggests that stabilizing the secondary structure formed in this region would be expected to reduce HER-2/neu transcription to a similar degree. In summary, these data suggest that in breast cancer cells, stabilizing the T:H structure in the GGA repeat region of the HER-2/neu promoter would be expected to reduce HER-2/neu expression by approximately 50%.

HER-2/neu Promoter-Reporter Cell Lines

We have also begun to create reporter cell lines in ZR-75-1 breast cancer cells and 293 cells. In these reporter cell lines, the host cells are genomically modified to contain a single copy of the flp recombinase target sequence (FRT). When a gene of interest is placed in a plasmid containing a FRT and co-transfected with a flp recombinase expression vector, the gene of interested is inserted into the FRT of the host cells by recombination, resulting in a homogenous population of cells that bear a single integrant of the gene of interest at the same genomic locus in the entire cell population.

This flp recombinase system is ideal for creating reporter cell lines bearing the HER-2/neu promoter driving the luciferase gene, because the reporter cells created with the wild-type HER-2/neu promoter-luciferase construct and the reporter cells created with the mutant HER-2/neu promoter-luciferase construct are completely isogenic, both bearing a single, integrated copy of the promoter-reporter construct at the same genomic location (the FRT site) in the host cells. In order to create an FRT site in the genome of the ZR-75-1 cells, we have begun stable selection of ZR-75-1 cells transfected with the FRT-lacZeo construct. When stable transfectants have been obtained and the presence of a single integrated FRT site confirmed by Southern blot, the ZR-75-FRT cells can be used to create isogenic cell lines containing the HNP386 (wild-type) and HNP386(Δ-225) promoter-luciferase constructs integrated at single copy into identical genomic locations. Thus, luciferase activity from these cell lines will reflect HER-2/neu promoter activity, and differences in luciferase activity between the resulting ZR-75-HNP386luc and ZR-75-HNP(Δ-225)luc cells will reflect the differences in HER-2/neu promoter activity imparted by the Δ-225 mutation. Moreover, the relative changes in luciferase activity following treatment of the cells with G-quadruplex binding compounds can be used to select compounds that are able to bind to the T:H secondary structure found in the HER-2/neu promoter. For example, our preliminary data demonstrate that telomestatin and several other lead compounds from Cylene, Inc. can bind to the T:H structure in the HER-2/neu promoter in cell-free DNA polymerase arrest assays. Treatment of ZR-75-HNP386luc and ZR-75-HNP(Δ-225)luc cells will be expected to result in inhibition of luciferase activity in the ZR-75-HNP386luc cells but not the ZR-75-HNP(Δ-225)luc cells. These cells can further be used for screening of additional compounds.

We are also constructing HER-2/neu promoter-reporter cell lines in 293-flp cells, which are commercially available cells already bearing the FRT recombination target sequence stably integrated at single copy into a unique genomic locus.

Examination of HIF-1α as an Alternate Therapeutic Target

We investigated the concept of inhibiting gene expression by stabilizing G-quadruplex or related DNA secondary structures in the HIF-1α gene as an additional or alternate therapeutic target. The rationale for studying HIF-1α derives from several key considerations:

1) HIF-1α is an important transcription factor that stimulates tumor cell growth and metastases due by controlling the expression of several pathways, including angiogenis, metabolism, invasion, and inhibition of apoptosis. In breast cancer, HIF-1α overexpression is seen immunohistochemically in 2 patterns: directly around necrosis, which is related to hypoxia and in a diffuse pattern. Breast cancer patients with perinecrotic HIF-1α overexpression have a poor prognosis and activate downstream targets of HIF-1α (such as Glut-1 and VEGF). The diffuse HIF-1α overexpression can also lead to downstream activation of HIF-1α target genes in breast cancer, particularly when p300 and p53 are also overexpressed, confirming the biologic and clinical impact of HIF-1 downstream activation (*14*).
2) HIF-1α has now become a very important therapeutic target for the development of experimental cancer therapeutics in a number of cancers, including breast cancer, and several investigative groups in academia and industry have drug screening programs for the discovery of HIF-1α inhibitors (*15*). However, these drug screening programs are hindered by the prevailing concept that HIF-1α itself, like many other important transcription factors, is not a "druggable" therapeutic target. Thus, efforts have been directed toward discovery of drugs that lead to HIF-1α inhibition by pathway inhibition, either upstream or downstream of HIF-1α, and several novel compounds are now in development that can accomplish this goal (*16*). The development of a small molecule that could inhibit HIF-1α at the transcriptional level thus enables the possibility of discovery of a direct HIF-1α inhibitor.
3) We discovered that the HIF-1α promoter has a G-rich region that shares high homology with a well-characterized G-quadruplex forming region in the c-myc promoter (*17*).
4) The ambiguities in the biochemical characterization of the unusual DNA secondary structure in HER-2/neu in the present work motivated us to examine a novel gene target, but one that we predicted to conform to a more thoroughly characterized DNA secondary structure.

We identified a G-rich sequence in the HIF-1α promoter bearing a sequence capable of forming a G-quadruplex (**Figure 3**). We demonstrated that the polypurine:polypyrimidine tract was required for constitutive transcription of the HIF-1α gene, and showed that the polypurine strand can fold into an intramolecular G-quadruplex to produce a potassium-dependent DMS footprint and a characteristic circular dichroism spectrum indicative of a parallel arrangement of the four strands involved in tetraplex formation. We showed that two G-quadruplex ligands, telomestatin and TmPyP4, are capable of binding to and stabilizing the G-quadruplex formed by the HIF-1α polypurine sequence. These data demonstrated that the G-quadruplex was composed of three stacked tetrads derived from a primary DNA sequence with the general motif: $G_3N_1G_3N_6G_3N_1G_3$. A sequence derived

from the c-myc oncogene, myc-1245, bears the same general sequence motif and was structurally characterized by nuclear magnetic resonance (NMR) as a parallel, propeller-type G-quadruplex composed of three stacks of G-tetrads and three double-chain-reversal loops (*18*). Based on these observations, we concluded that the HIF-1α promoter forms a parallel-propeller G-quadruplex, and suggested that other genomic sequences bearing the general sequence motif of $G_3N_1G_3N_xG_3N_1G_3$ (x = 3-9) could probably also form this stable DNA secondary structure. Of note, the HIF-2α promoter (*17*) and the VEGF promoter (*19*) also contain similar cis-regulatory elements bearing this common structural motif, and thus might thus be suppressed by G-quadruplex binding drugs to inhibit tumor angiogenesis and the adverse tumor biology that occurs due to the activation of HIF-α target genes. These data were published in *Biochemistry* in 2005.

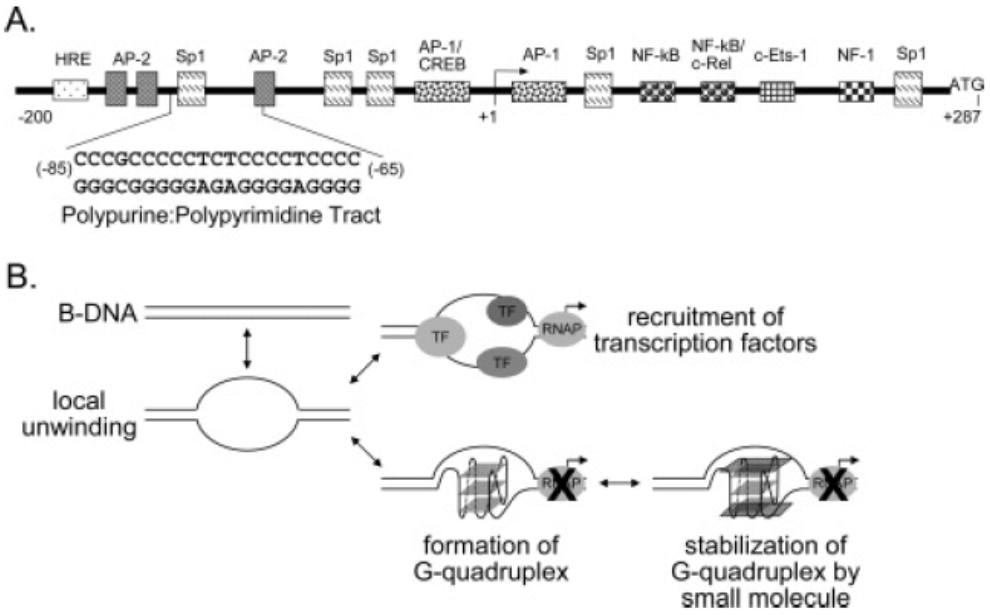

Figure 3. (A) Schematic representation of the minimal HIF-1α promoter highlighting putative transcription factor binding site assignments and the polypurine/polypyrimidine tract. **(B)** Model of promoter silencing by G-quadruplex formation in the HIF-1α promoter.

Key Research Accomplishments

Pre-award through End of Year 1

- Identification of a GGA repeat region of the HER-2/neu promoter capable of forming an unusual secondary structure, a tetrad-heptad, related to G-quadruplex.
    - Biochemical characterization of secondary structure formation using oligonucleotides representing the HER-2/neu promoter.
- Identification of two classes of compounds (telomestatin, a natural product, and a proprietary fluoroquinolone from Cylene Pharmaceuticals) capable of binding to the tetrad :heptad structure in the HER-2/neu promoter using a cell-free biochemical assay (DNA polymerase arrest assay)

- Preliminary demonstration that compounds identified in the cell-free assay can reduce HER-2/neu transcription *ex vivo* (in cell culture)

Year 2

- Construction of reporter plasmids bearing the HER-2/neu promoter and specific mutations that disrupt secondary structure formation in the HER-2/neu promoter.
  - Transient transfection analysis of native and mutant HER-2/neu reporter plasmids.
- Initiation of the construction of reporter cell lines for evaluation of the biological role of secondary structure formation in HER-2/neu expression and identification of compounds that can specifically inhibit HER-2/neu expression by binding to the secondary structure formed in the HER-2/neu promoter
- Identification of a G-quadruplex forming region of the HIF-1α promoter.
  - Biochemical characterization of secondary structure formation using oligonucleotides representing the HIF-1α promoter.
  - Identification of two classes of compounds (telomestatin, a natural product, and TmPyP4, a cationic porphyrin) that can bind to the G-quadruplex formed by the HIF-1α promoter.

Reportable Outcomes (reprints, presentations, patents, etc.)

Meeting Abstracts

1. Y. Krotova, M. Boros, R. Memmott, A. Ziemba, L. Hurley, and S. Ebbinghaus, Transcriptional Control of the HER-2/neu Promoter By DNA Secondary Structure, Era of Hope Department of Defense Breast Cancer Research Program Meeting, 2005.

2. De Armond RL, Deniro M, Hurley L, **Ebbinghaus SW**. Evidence for a G-quadruplex in the promoter region of the hypoxia inducible factor-1 alpha (HIF-1α). *Proceedings of the American Association for Cancer Research,* Volume 45, 2004 (Abstract 3863).

Peer-reviewed Manuscript

1. De Armond R, Wood S, Sun D, Hurley LH, **Ebbinghaus SW**. Evidence for the Presence of a Guanine Quadruplex Forming Region within a Polypurine Tract of the Hypoxia Inducible Factor 1α Promoter. *Biochemistry* 44(49):16341-50, 2005.

Conclusions

We conclude that the GGA repeats in the HER-2/neu promoter can form a stable secondary structure known as a tetrad:heptad, although the biochemical characterization of the secondary structure formed by the full length of the HER-2/neu PPT has proven difficult because of competing secondary structures by these G-rich oligos. Nonetheless, we have demonstrated in principle that we can use a simple DNA polymerase arrest assay to screen compounds that might be useful inhibitors of HER-2/neu transcription, and using this assay have identified test compounds that can be used as leads for the development of small molecule inhibitors of HER-2/neu transcription with therapeutic intent. Our work in progress as this annual report is prepared will lead to the development of a cell-based biological assay based on reporter cell lines with native and T:H mutant

HER-2/neu promoters that can be used to corroborate the cell-free assay data derived from the DNA polymerase arrest assay and identify compounds capable of binding inhibiting HER-2/neu transcription by interacting with the secondary structure formed by the HER-2/neu promoter. These reporter cell lines will also be critical for clarifying the biological role of this region of the HER-2/neu promoter and can be used for proving the existence of a G-quadruplex related structure in living cells.

Furthermore, we have discovered another example of an important therapeutic target for breast cancer drug development, HIF-1α, which has a critical cis-regulatory element capable of forming a G-quadruplex that can be bound by G-quadruplex binding small molecules.

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Appendix
Reprint of publication

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Evidence for the Presence of a Guanine Quadruplex Forming Region within a Polypurine Tract of the Hypoxia Inducible Factor 1α Promoter†

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ABSTRACT: The promoter of the hypoxia inducible factor 1 alpha (HIF-1α) gene has a polypurine/polypyrimidine tract (−65 to −85) overlapping or adjacent to several putative transcription factor binding sites, and we found that mutagenesis of this region diminished basal HIF-1α expression. Oligonucleotides representing this region of the HIF-1α promoter were analyzed by electrophoretic mobility shift, chemical probing, circular dichroism, and DNA polymerase arrest assays. The guanine-rich strand was found to form a parallel, unimolecular quadruplex in the presence of potassium that was further stabilized by two known quadruplex binding compounds, the cationic porphyrin TmPyP4 and the natural product telomestatin, while TmPyP2, a positional isomer of TmPyP4, did not stabilize quadruplex formation. These data suggest that a quadruplex structure may form in a region of the HIF-1α promoter that regulates basal HIF-1α expression.

Hypoxia inducible factor 1 (HIF-1)[1] is a transcription factor composed of a hypoxia inducible alpha subunit (HIF-1α) and a constitutively expressed beta subunit (HIF-1β) responsible for the regulation of over 60 genes involved in oxygen homeostasis (*1*, *2*). HIF-1α is overexpressed in many common human tumors as a result of intratumoral hypoxia (*3*). HIF-1α levels are normally undetectable in normoxia due to posttranslational processing involving proline hydroxylation and the von Hippel Lindau (VHL) protein, a multifunctional adapter molecule that mediates the ubiquitinylation of HIF-1α, targeting it to the proteasome for degradation (*2*). During hypoxia, HIF-1α accumulates, translocates to the nucleus, and dimerizes with HIF-1β. The HIF-1 heterodimer then binds to a DNA consensus sequence known as the hypoxia response element (HRE) (*4*). In tumors, the activation of hypoxia responsive genes leads to angiogenesis, metabolic adaptation, resistance to apoptosis, and the expression of a variety of genes associated with local invasion or metastasis (*5*, *6*). The vascular endothelial growth factor (VEGF) gene is an example of a gene activated by HIF-1. VEGF is a pro-angiogenic ligand secreted by many tumors that binds to tyrosine kinase receptors (VEGF-R1 and -R2) expressed predominantly on angioblasts and endothelial cells, recruiting these cells to form neovessels in the tumor (*7*).

HIF-1α may be abnormally overexpressed in the absence of hypoxia by several mechanisms, such as the loss of the VHL tumor suppressor gene, a frequent event in renal cell carcinoma that disrupts the usual posttranslational regulation of HIF-1α in normoxia (*8*). HIF-1α levels may also be controlled under normoxia by the rate of HIF-1α translation in certain types of cells in response to hormones, growth factors, or cytokines. This alternate pathway of HIF-1α regulation appears to involve signaling through the phosphatidyl inositol 3 kinase (PI3K) pathway, resulting in the activation of the p70S6K translation factor through mTOR (molecular target of rapamycin) and involves the recognition of a 5′-terminal oligopyrimidine tract (5′-TOP) in the HIF-1α mRNA (*9*−*15*).

In most cells, HIF-1α mRNA levels do not increase in hypoxia compared to normoxia, but elevated levels of HIF-1α mRNA can be detected in some human tumor specimens or induced by hypoxia in certain mammalian cells (*12*, *16*−*21*). Transcriptional upregulation of HIF-1α gene expression is another mechanism of inducing HIF-1α expression by nonhypoxic stimuli in certain cell types, such as angiotensin II in vascular smooth muscle cells (*12*) and lipopolysaccharide in macrophages (*22*). Transcriptional upregulation of HIF-1α expression by angiotensin II was shown to require the presence of the untranslated region (5′UTR) of the HIF-1α gene and involve signaling through the protein kinase C (PKC) pathway (*12*). Human and murine HIF-1α promoters are highly conserved (*23*), and HIF-1α transcription is predominantly controlled by a 200 base pair (bp) core promoter upstream of the transcription start site and the 287 bp 5′UTR (*24*). Functional elements of the HIF-1α promoter were identified by transient expression of reporter gene constructs containing serial 5′ deletions in normoxic and cobalt chloride treated endothelial and tumor cell lines (*24*).

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[1] Abbreviations: HIF-1α, hypoxia inducible factor 1 alpha; VHL, Von Hippel Lindau; HRE, hypoxia response element; VEGF, vascular endothelial growth factor; PI3K, phosphatidyl inositol 3 kinase; mTOR (molecular target of rapamycin; 5′ TOP, 5′ terminal oligopyrimidine tract); 5′UTR, 5′ untranslated region; PKC, protein kinase C; G-quadruplex, guanine quadruplex; PPT, polypurine tract; TmPyP2, 5,-10,15,20-tetra(*N*-methyl-2-pyridyl)porphin; TMPyP4, 5,10,15,20-tetra-(*N*-methyl-4-pyridyl)porphin; ODNs, oligodeoxyribonucleotides; DMS, dimethyl sulfate; CD, circular dichroism.

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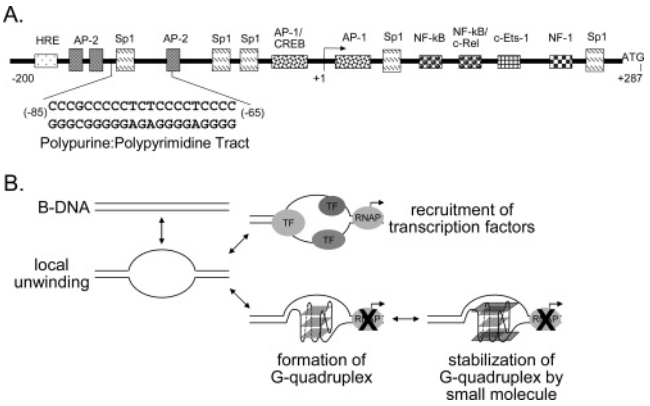


FIGURE 1: (A) Schematic representation of the minimal HIF-1α promoter highlighting putative transcription factor binding site assignments and the polypurine/polypyrimidine tract. (B) Model of promoter silencing by guanine quadruplex formation.

The human HIF-1α promoter lacks a TATA box, but contains putative binding sites for Sp1, NF-1, AP-1, AP-2, and HIF-1 upstream of the transcription initiation site as well as several putative transcription factor binding sites in the 5′UTR (*23*, *24*).

The critical cis-acting elements involved in the transcriptional upregulation of HIF-1α in hypoxia are located in the first 100 bp of the HIF-1α promoter (*24*). Within this region, there is a mostly uninterrupted polypurine:polypyrimidine tract from −85 to −65 upstream of the transcription start site and overlapping putative Sp1 and Ap-2 binding sites (Figure 1). Guanine-rich sequences can associate into four-stranded structures formed from stacks of guanine tetrads, and sequences conforming to a motif potentially capable of forming such structures have been reported to be relatively common in the human genome (*25*−*29*). The Hoogsteen-bonding of the guanine tetrads is favored by the presence of a monovalent cation, especially potassium, which fits within a central core formed by the carbonyl groups of the guanines (*30*, *31*). Intermolecular guanine quadruplexes can form from the association of two or four strands of DNA (or RNA), and such structures may form under physiological conditions by the human immunodeficiency virus RNA and by some guanine-rich aptameric oligonucleotides (*32*−*39*). Intramolecular guanine quadruplexes form by the folding of a single strand, and guanine-rich sequences that are potentially capable of forming these structures can be found in telomeric DNA from humans, most eukaryotes, and several lower organisms (*40*−*49*). Sequences derived from the human immunoglobin switch region, insulin gene, and fragile X syndrome gene are also capable of forming quadruplexes (*50*−*53*). The DNA helicases that are deficient in Bloom's syndrome and Werner's syndrome can recognize quadruplex DNA, suggesting that these structures form in cells and if not resolved lead to difficulties during DNA recombination (reviewed in ref *54*). Recently, guanine-rich sequences from a nuclease hypersensitivity element of the human c-myc oncogene promoter were shown to form several different quadruplex structures and appear to be important in regulating c-myc transcription (*55*−*58*). Additional evidence for G-quadruplexes in the regulatory regions of human genes was reported for three muscle-specific genes (*59*). The human telomeric G-quadruplex has been studied as a target for drug development for the treatment of cancer, and several DNA binding compounds have a preference for quadruplex DNA (*60*−*62*). Silencing gene expression by ligand interaction with a G-quadruplex in a gene promoter is illustrated in Figure 1 and has been described for the c-myc oncogene. Molecules capable of binding to the G-quadruplexes formed by the c-myc promoter include TmPyP4, a cationic porphyrin; 307A, a 2,6-pyridin-dicarboxamide derivative; Hoechst 33258; and telomestatin, a natural product isolated from *Streptomyces anulatus* and previously shown to be a potent telomerase inhibitor (*58*, *63*−*66*).

In the present work, we wished to investigate the importance of the polypurine tract (PPT) in the HIF-1α promoter to HIF-1α transcription and determine whether the guanine-rich strand of this element could form an intramolecular quadruplex. Substitution mutations in the PPT markedly diminished basal HIF-1α expression in Caki-1 (VHL wild-type kidney cancer) cells. Dimethyl sulfate footprinting and circular dichroism studies of oligonucleotides representing the guanine-rich strand of the HIF-1α PPT suggest the formation of a unimolecular quadruplex in the presence of potassium, and DNA polymerase arrest assays show stabilization of the quadruplex with TmPyP4 and telomestatin. The solution structure for the c-myc quadruplex has been recently reported (*67*, *68*), and based on the presence of similar $G_3NG_3N_6G_3NG_3$ sequence motifs in the quadruplex forming regions of both the HIF-1α promoter and the more thoroughly studied c-myc promoter, we suggest that a similar structure forms in the HIF-1α promoter and may play a role in regulating HIF-1α gene expression.

MATERIALS AND METHODS

Chemical Reagents and Oligonucleotides. 5,10,15,20-Tetra(*N*-methyl-2-pyridyl)porphin (TMPyP2) and 5,10,15,-20-tetra(*N*-methyl-4-pyridyl)porphin (TMPyP4) were purchased from Mid-Century Chemicals (Posen, IL). Telomestatin was a gift from Dr. Kazuo Shin-ya (University of Tokyo, Japan). Oligodeoxyribonucleotides (ODNs) were purchased from commercial vendors and gel purified. Concentrations were determined by absorbance measurements at 260 nm using the weighted sum method to calculate the molar extinction coefficient for each ODN according to its composition (*69*). Calculated extinction coefficients for the ODNs are presented as Supporting Information. The sequences of the ODNs used in these studies are given in Table 1 and shown schematically in Figure 3.

Electrophoretic Mobility Shift Assays. PAGE-purified oligonucleotides were end-labeled by the T4 polynucleotide kinase reaction, purified on spin columns, and eluted into pure water. In the experiment illustrated in Figure 5A, G-quadruplex formation was performed by diluting the ODNs to a final concentration of 0.1 μM in a buffer containing 50 mM Tris-Cl (pH 7.6) with or without 140 mM KCl, heating the solution to 95 °C for 5 min and then slow cooling to room temperature over 3−4 h. The solutions were weighted by adding 50% glycerol (v/v, without dyes) to a final concentration of 5% and loaded onto a 12% nondenaturing, high-potassium polyacrylamide gel containing 90 mM Tris-Cl, 90 mM borate, 1 mM EDTA, and 140 mM KCl (pH 8.0). The electrophoresis was performed at room temperature at 10 V/cm for 6 h, and the electrophoresis running buffer contained 90 mM Tris-Cl, 90 mM borate, 1 mM EDTA (1× TBE) without KCl. In the experiment

Table 1: Oligonucleotide Sequences[a] and Summary of DMS Footprinting and CD Spectroscopy

ODN	Sequence (5′→3′)	DMS Footprint	CD 260 nm peak
I	CGCGCTCCCGCCCCCTCTCCCCTCCCCGCGC	no	no
II	GCGCGGGGAGGGGAGAGGGGGCGGGAGCGCG	yes	yes
III	GGGGAGGGGAGAGGGGGCGGGA	yes	yes
IV	**TCGGGC-** GCGCGGGGAGGGGAGAGGGGGCGGGAGCGCG	yes	no
V	**CAGGGGGCGGGCAAGGGCGGAGGCGCGCTCGGGC**	no	ND
VI	**CAGGGGGCGGGCAAGGGCGGAGGCGCGCTCGGGC-** GCGCGGGGAGGGGAGAGGGGGCGGGAGCGCG	yes	ND
VII	GCGCGGGGAGGGGAGAGGGGGCGGG**ga**CGCG	yes	yes
VIII	GCGCGG**c**GAG**c**GGAGAGGG**c**GCG**c**GAGCGCG	no	no
IX	GCGCGGGGAGGG**ctctcc**GGGCGGGAGCGCG	no	no
X	GCGCGGGGAGGGG**tct**GGGGGCGGGAGCGCG	yes	yes
XI	GCGCGGGGAGGG**ctct**GGGGGCGGGAGCGCG	weak	no
XII	GCGCGGGGAGGGG**tctc**GGGGCGGGAGCGCG	yes	yes
XIII	GCGCGGGGAGGGG**tctcc**GGGCGGGAGCGCG	weak	no
XIV	GCGCGGGGAGGG**ctctc**GGGGCGGGAGCGCG	weak	no
myc1245	**t**GGGGAGGG**ttttta**GGG**t**GGG**ga**	yes	ND
polP	TAATACGACTCACTATAGCAATTGC		
polH	TCCAACTATGTATACGCGCGGGGAGGGGAGAGGGGCGGGAGCGCGTTAGCGACACGCAATT*GCTATAGTGAGTCGTATTA*		
polC	TCCAACTATGTATACTACGTATTCAGCTCATGCTATACGTCATCGTTAGCGACACGCAATT*GCTATAGTGAGTCGTATTA*		

[a] Polypurine sequences are aligned with ODN II where applicable. Bold uppercase sequence represents the sequence adjacent to ODN II in the HIF-1α promoter. Bold lowercase nucleotides represent substitutions in ODN II. The DNA polymerase arrest templates contain the sequence of ODN II (underlined in polH) or a random control sequence (underlined in polC) upstream of the polP primer binding site (italics). ND, not done.

illustrated in Figure 5B, G-quadruplex formation was performed by diluting ODN II to a final concentration of 0.1 μM in a buffer containing 10 mM Tris-Cl (pH 7.4) plus either 140 mM KCl, 140 mM NaCl, or no additional salt. The solutions were heated to 95 °C for 5 min, slowly cooled to 37 °C over 2−3 h, and then DMS was added to a final concentration of 0.25%. The solutions were weighted with glycerol, and immediately loaded onto a 10% nondenaturing, low-potasium polyacrylamide gel buffered with 1× TBE plus 10 mM KCl, resulting in a total DMS incubation time of under 5 min. Electrophoresis was performed at room temperature at 10 V/cm for 6 h followed by autoradiography. A separate set of samples was handled identically except that DMS was not added, demonstrating that the DMS treatment did not alter the electrophoretic mobility of ODN II (not shown). The bands indicated in Figure 5B were excised from the gel, and the DMS treated ODN was eluted in gel elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA, pH 8.0) at 37 °C for 4 h. The gel eluate containing the DMS treated ODN was concentrated and washed using Sep-Pak C18 columns, eluted from the columns in 60% methanol and lyophilized. The DMS treated ODNs were then dissolved in 10% piperidine for piperidine cleavage as described for DMS footprinting.

DMS Footprinting. The dimethyl sulfate (DMS) footprints for G-quadruplexes were performed using end-labeled ODNs according to the chemical sequencing method of Maxam−Gilbert essentially as described (*63*). G-quadruplex formation was performed as described above in 50 mM Tris-HCl (pH 7.6) with or without 140 mM KCl by heating the ODNs to 95 °C and slowly cooling to reoom temperature over 3−4 h. The solutions were treated with 0.25% DMS for 5 min. DMS stop solution [1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, 250 μg/mL tRNA] was added, the ODNs were precipitated, treated with 10% (v/v) piperidine, evaporated to dryness, redissolved in formamide loading buffer, and loaded onto a 12% polyacrylamide sequencing gel.

DNA Polymerase Arrest Assay. The DNA polymerase arrest assay was performed by modification of previously reported methods (*70*, *71*). A 25-mer taq polymerase primer (polP) was end-labeled, and a 2-fold excess of primer was annealed to 0.5 pmol of taq polymerase template (polH or polC). The resulting asymmetric primer-template duplex was gel purified and eluted in 2× taq polymerase arrest assay buffer [120 mM Tris-HCl (pH 8.5), 30 mM ammonium sulfate, 15 mM $MgCl_2$]. The arrest assay was performed in a buffer of 60 mM Tris-HCl (pH 8.3), 15 mM ammonium sulfate, 7.5 mM $MgCl_2$, 1.5 mM dNTPs, 0 to 140 mM KCl, in a final volume of 10 μL. Primer-template duplex in the arrest assay buffer was incubated at 37 °C with 0−1 μM of TmPyP4, TmPyP2, or telomestatin. Reaction mixtures were brought to 47 °C, primer extension was initiated by adding 5 U of taq DNA polymerase (Fermentas, Hanover, MD), and the reaction was incubated at 47 °C for 20 min. Primer extension was stopped with 10 μL taq polymerase stopping buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% xylene cylanol, 0.1% bromophenol blue), denatured at 95 °C for 5 min, quickly cooled on ice, and primer extension products were separated on 10% sequencing gels. The arrest sites were identified by alignment with a chain termination

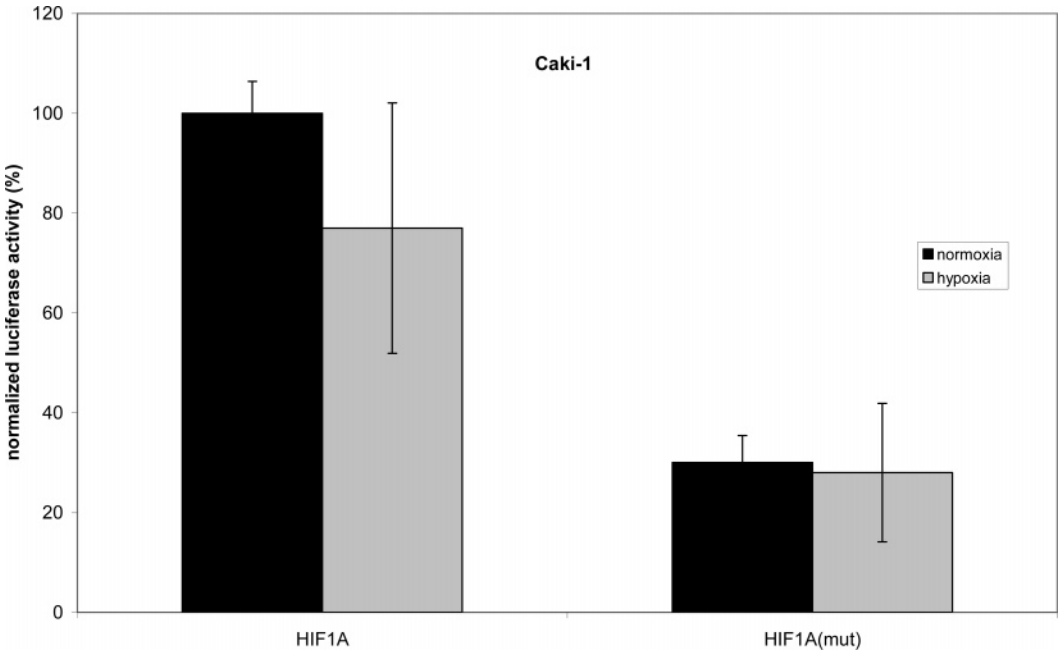


FIGURE 2: Transient transfection analysis shows that disruption of the PPT by substitution mutations diminishes HIF-1α expression in normoxia and hypoxia. Transfections were performed in duplicate, and the data represent the mean (± standard deviation) of normalized luciferase activity from three independent experiments.

sequencing reaction using the same primer−template duplex with the Thermosequenase kit (USB Corporation, Cleveland, OH) according to the manufacturer's instructions.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured on ODNs at a concentration of 100 μM in either 50 mM Tris-HCl, 25 mM NaCl, or 25 mM KCl. Spectra were measured on a Jasco-810 spectropolarimeter (Jasco, Easton, MD) using a quartz cell of 1-mm optical path length, an instrument scanning speed of 100 nm/min, with a response of 1 s, over a range of 200 to 350 nm. A set of three scans was averaged for each sample at 25 °C.

Transient Transfections. The plasmid pGL3/HIF1A was constructed as follows. The HIF-1α promoter from −538 to +291 (relative to the transcription start site) was amplified by polymerase chain reaction (PCR) from pooled human genomic DNA (Boehringer-Mannheim) using primers (forward: GAACAGAGAGCCCAGCAGAGTTGGGCGG and reverse: CCTCCATGGTGAATCGGTCCCCGCGATG) that were previously described (*24*). The amplicon was cloned into the pCR2.1-TOPO vector (Invitrogen), excised, ligated upstream of the luciferase gene in the pGL3 Basic vector (Promega), and the sequence of the insert of the resulting pGL3/HIF1A construct was confirmed. The PPT was disrupted in the plasmid pGL3/HIF1A^{mut} by multiple (fifteen) substitution mutations from −84 to −68 introduced by site-directed mutagenesis (top strand: CCGCCCCCTCTCCCCTC → GAATTCCTCGAGGAGCT; site-directed mutagenesis and sequencing were performed by Topgene, Canada). Caki-1 (VHL+/+ kidney cancer) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown under standard conditions. The transfection mixture for Caki-1 cells included 2 μg of pGL3/HIF1A or pGL3/HIF1A^{mut}, 20 ng pRL/SV40 (Promega), and 4 μL LT-1 (Mirus, Madison, WI). Cells at 70% confluence were transfected in RPMI for 2 h at 37 °C in normoxia, and then cells were incubated in either normoxia or hypoxia (1% O_2) at 37 °C for 24 h. Dual luciferase assays were performed with commercial reagents (Biotium, Hayward, CA) according to the manufacturer's instructions. The ratio of firefly to renilla luciferase activity was calculated, and normalized for the average firefly/renilla luciferase activity for pGL3/HIF1A in normoxia for each experiment. Transfections were performed in duplicate and repeated three times. The data presented in Figure 2 represent the mean (± standard deviation) normalized luciferase activity.

RESULTS

To determine whether the HIF-1α PPT was needed for basal HIF-1α transcription, we performed transient transfections on wild-type and PPT mutant HIF-1α promoters in Caki-1 cells. As shown in Figure 2, HIF-1α transcription is not induced by hypoxia in Caki-1 cells. The PPT was disrupted in pGL3/HIF1A^{mut} by introducing 15 substitution mutations between −84 to −68. Disrupting the PPT caused a 70% decline in HIF-1α expression both in normoxia and hypoxia in Caki-1 cells (Figure 2).

To determine whether the HIF-1α PPT could undergo a conformational shift to a DNA secondary structure, we evaluated the series of oligonucleotides (ODNs) illustrated in Figure 3 which represent native or mutant sequences from the HIF-1α promoter. These ODNs were used in footprinting, electrophoretic mobility shift, and circular dichroism studies in solution.

Hoogsteen hydrogen bonds render the N-7 position of guanines in a tetrad inaccessible to dimethyl sulfate (DMS), and the guanines involved in G-quadruplex formation can be detected by DMS footprinting. In Figure 4, the DMS cleavage patterns of ODNs I, II, III, and VIII are shown in the presence and absence of 140 mM potassium ions and compared to a homologous sequence derived from the c-myc promoter, myc1245, for which the solution structure determined by NMR has been reported (*67*). In the presence of potassium, strong footprints are produced by ODN II, ODN

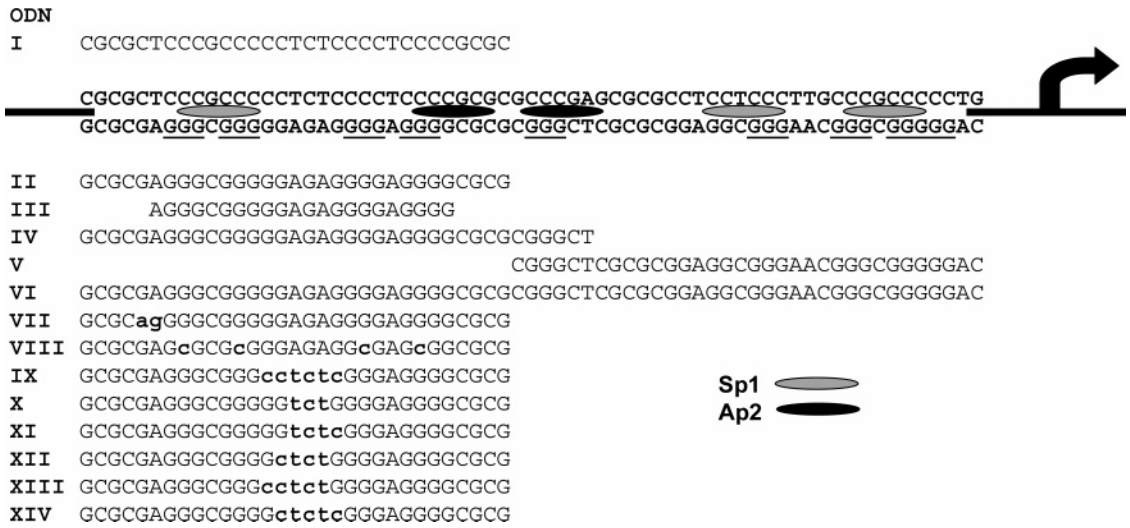


FIGURE 3: Oligonucleotides representing the HIF-1α promoter which were used to evaluate DNA secondary structure formation. ODN I is written in 5′ → 3′ orientation. ODNs II−XIV are written in 3′ → 5′ orientation and aligned with the bottom strand of the HIF-1α promoter. Mutated nucleotides are in lower case and bold. Runs of three or more guanines are underlined.

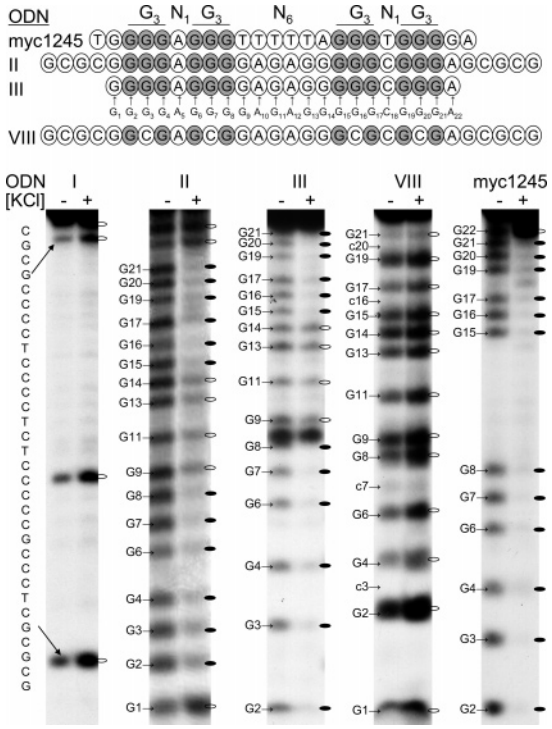


FIGURE 4: The sequences of oligonucleotides representing the quadruplex forming regions of the c-myc and HIF-1α promoters and conforming to the $G_3N_1G_3N_6G_3N_1G_3$ sequence motif are illustrated schematically. DMS footprinting reactions with ODNs representing the HIF-1α promoter in the absence (−) or presence (+) of 140 mM KCl in comparison with the footprint produced by the myc1245 ODN. In the autoradiograms, the guanines of the guanine-rich ODNs are numbered in alignment with ODN III as shown schematically above the autoradiograms. Protected guanines are indicated by closed ovals and unprotected guanines are indicated by open ovals, and the positions of the G → C substitutions in ODN VIII are indicated.

III, and myc1245. The footprints are characterized by the protection of four runs of three contiguous guanines and are consistent with the formation of a guanine quadruplex composed of three stracks of guanine tetrads. When the sequences are numbered from the 5′ end of ODN III (representing the beginning of the first run of guanines and corresponding to position −65 in the HIF-1α promoter), DMS protection is observed at G2-G4, G6-G8, G15-G17, and G19-G21. As one would expect, guanines 5′ and 3′ to the quadruplex forming region of the PPT, G1, G23, and G25 in ODN II, are reactive with DMS. In addition, four central guanines of ODN II and ODN III, G9, G11, G13, and G14, are also reactive with DMS. We interpret this pattern of DMS protection to be consistent with the formation of an intramolecular G-quadruplex composed of three stacked guanine tetrads in a configuration that places the central guanines in a loop composed of six nucleotides. ODN II and ODN III produced similar footprints, showing that the immediate flanking sequences are neither necessary nor detrimental to quadruplex formation. No protection from DMS methylation is seen for ODN I (the complementary sequence to ODN II). Similarly, ODN VIII (G → C substitution at the central guanine of each run of protected guanines) did not produce a DMS footprint, consistent with the lack of quadruplex formation when these runs of guanines are disrupted.

The folding of an ODN into an intramolecular quadruplex frequently makes the ODN more compact and migrate at a faster rate during gel electrophoresis, while the intermolecular association of multiple ODNs would be expected to result in complexes of slower mobility (*46*, *72*−*74*). In Figure 5A, we compare the electrophoretic mobility of the guanine-rich strand of the HIF-1α PPT with ODNs bearing mutations that disrupted G-quadruplex formation. The electrophoretic mobility of these ODNs was compared in a high-potassium gel, containing 140 mM KCl in the gel matrix to preserve the DNA secondary structures formed during incubation in 140 mM KCl prior to electrophoresis. ODN VIII (bearing four G → C substitutions) and ODN IX (bearing six mutations in the loop region) migrate as a single species in the gel, whether incubated in the presence or absence of 140 mM potassium before electrophoresis in the high-potassium gel. In contrast, ODN II forms multiple slower migrating species in the high-potassium gel that are consistent with the formation of bimolecular (2 stranded or 2s) and tetramolecular (4 stranded or 4s) G-quadruplexes. We demonstrated that the unimolecular G-quadruplex (1s) has the same electrophoretic mobility as the unstructured form of ODN

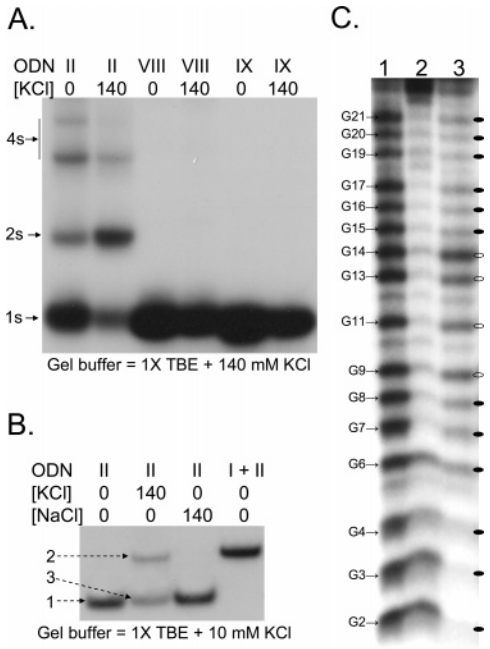
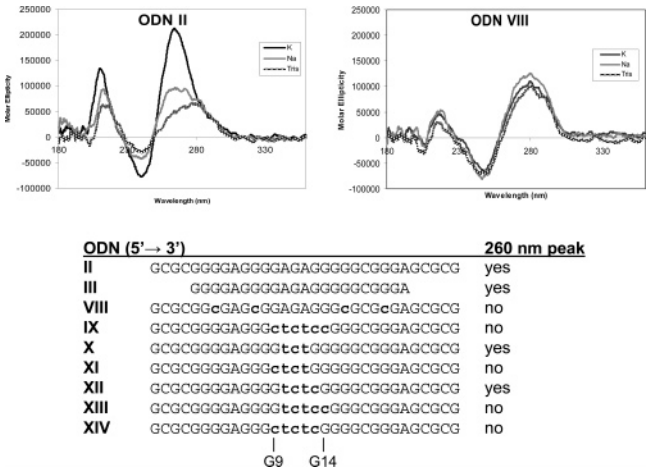


FIGURE 5: Electrophoretic mobility shift assays with the guanine-rich strand of the HIF-1α PPT. (A) ODN II migrates as unimolecular (1s), bimolecular (2s), and tetramolecular (4s) species in a high-potassium gel, while ODNs bearing substitution mutations in the runs of guanines (ODN VIII) or the loop region (ODN IX) migrate as a single, unimolecular species. (B) ODN II was incubated in 140 mM KCl, 140 mM NaCl, or no additional salt, and then DMS was added to each sample before loading onto a low-potassium (10 mM KCl) gel. Duplex DNA composed of ODN I + II was included for comparison as a size marker. After electrophoresis, the bands indicated by arrows were excised from the gel, and the ODNs were subjected to piperidine cleavage. (C) The piperidine cleavage reactions were resolved on a 10% sequencing gel. Protected guanines from the unimolecular species of ODN II with KCl from band 3 (in lane 3) are denoted by closed ovals while reactive guanines are denoted by open ovals, and this characteristic footprint demonstrates that the intramolecular G-quadruplex formed ODN II has the same electrophoretic migration as the unstructured form of the HIF-1α PPT.



ODN (5'→ 3')		260 nm peak
II	GCGCGGGGAGGGGAGAGGGGGCGGGAGCGCG	yes
III	GGGGAGGGGAGAGGGGGCGGGA	yes
VIII	GCGCGG**c**GAG**c**GGAGAGGG**c**GCG**c**GAGCGCG	no
IX	GCGCGGGGAGGG**ctctcc**GGGCGGGAGCGCG	no
X	GCGCGGGGAGGGG**tct**GGGGGCGGGAGCGCG	yes
XI	GCGCGGGGAGGG**ctct**GGGGGCGGGAGCGCG	no
XII	GCGCGGGGAGGGG**tctc**GGGGCGGGAGCGCG	yes
XIII	GCGCGGGGAGGGG**tctcc**GGGCGGGAGCGCG	no
XIV	GCGCGGGGAGGG**ctctc**GGGGCGGGAGCGCG	no

G9 G14

FIGURE 6: Circular dichroism spectroscopy with ODNs representing the PPT of the HIF-1α promoter. Spectra for ODN II, the native HIF-1α promoter sequence, and ODN VIII bearing mutations in the central guanines of each run are shown. Data for a series of ODNs lacking flanking sequence (ODN III) or bearing mutations in the loop region (ODNs IX−XIV) are summarized as either producing or lacking the characteristic potassium-dependent peak at 260 nm seen with ODN II. Loop mutants with disruption of G9 or G14 destabilize G-quadruplex formation.

II by treating the G-quadruplex reactions with DMS just prior to electrophoresis in a low-potassium gel (Figure 5B, containing 10 mM KCl in the gel matrix, a minimal concentration intended to preserve the most stable secondary structures formed before electrophoresis), followed by band excision, and piperidine cleavage to generate the DMS footprints shown in Figure 5C. In the absence of potassium before electrophoresis, ODN II migrates as a single species in the low-potassium gel (band 1) which does not produce a footprint. When ODN II is incubated with 140 mM potassium before electrophoresis, and bands comigrating with double-stranded ODN I + II (band 2) and single-stranded ODN II (band 3) are excised from the gel, DMS protection consistent with G-quadruplex formation is observed. The unimolecular G-quadruplex (band 3) comigrates with unstructured ODN II and produces a footprint identical to the footprints shown in Figure 4. The potassium-dependent slower mobility species in Figure 5B (band 2) is shown to be a bimolecular G-quadruplex, since it comigrates with duplex DNA (formed by annealing ODN II to its complement, ODN I) and produces an extended DMS footprint that includes the central guanines in the sequence. The slowest migrating potassium-dependent species formed by ODN II in Figure 5A (4s) are most likely tetramolecular G-quadruplexes, and they also produce an extended footprint that includes the central guanines (not shown). Interestingly, sodium ions do not appear to support stable G-quadruplex formation with this sequence under these conditions, since ODN II incubated in 140 mM sodium ions before electrophoresis migrates as a single species (Figure 5B) which does not produce a DMS footprint when excised from the gel (not shown).

G-quadruplexes have been probed by circular dichroism to deduce the orientation of the strands, because the parallel and antiparallel arrangement of the strands usually show characteristic spectra (*39*, *75*−*78*). The CD spectra of ODN II and VIII are compared in Figure 6. In potassium, ODN II produces a strong CD maximum at ∼260 nm, which is consistent with a parallel orientation of the strands in the G-quadruplex. In Tris buffer, the CD peak for ODN II widens and shifts to 280 nm, while in sodium, the CD spectrum appears to be intermediate between the spectrum for ODN II in potassium and ODN II in Tris buffer in the absence of a monovalent cation. ODN VIII, bearing a mutation in the central guanine of each run involved in G-quadruplex formation, produces a much different CD spectrum, with maxima at 280 nm in potassium, sodium, and Tris without monovalent cation. ODN III, lacking the flanking sequences outside the G-quadruplex forming region, yielded similar CD spectra to ODN II, with a positive peak at 260 nm, again consistent with the formation of a parallel G-quadruplex.

We evaluated a series of ODNs with alterations (G to C or A to T inversions) in the nucleotides (nt) of the predicted central loop of the G-quadruplex, summarized in Table 1. Collectively, these data demonstrated that alterations of G9, G14, or both tended to destabilize G-quadruplex formation. For example, ODN IX contains a 6 nt mutation of the entire loop region and does not produce the characteristic DMS footprint or CD spectrum of the HIF-1α G-quadruplex. ODN XI, XIII, and XIV, with 4−5 nt mutations starting at G9 or ending at G14, did not produce strong CD maxima at 260 nm and produced weak DMS footprints in potassium. ODN X and XII, with central 3−4 nt mutations produced strong

CD maxima at 260 nm and potassium-dependent DMS footprints similar to those shown for ODN II. These data demonstrate that the most central guanines in the loop, G11 and G13, do not participate in stabilizing the HIF-1α promoter G-quadruplex, but the two outer guanines in the loop, G9 and G14, may be needed for stable G-quadruplex formation. These observations are somewhat surprising in comparison to the myc1245 ODN, which has 6 nt substitutions in its central loop region, was shown elsewhere to form a stable G-quadruplex structure (*67*), and produced a strong DMS footprint in our studies. It is interesting to note that the guanine runs in myc1245 differ slightly from the runs of guanines in the HIF-1α ODNs by having runs of four guanines at both the 5′ and 3′ ends, while the HIF-1α ODNs lack the 3′ run of four guanines. Loop or flanking guanines may help to stabilize the G-quadruplex while not participating in the formation of the guanine tetrads.

We performed EMSA, DMS footprinting, and CD spectroscopy on several additional ODNs representing various segments of the HIF-1α PPT, summarized in Table 1. G-quadruplex forming sequences often occur within the context of a longer PPT. We wished to examine whether the polypurine sequences adjacent to the G-quadruplex forming sequence of the HIF-1α promoter were also capable of forming competing G-quadruplex structures using one or more additional runs of guanines. ODN V represents another PPT immediately downstream of the quadruplex forming sequence which also contains four runs of three or more contiguous guanines, albeit spaced at longer and less regular intervals. ODN V does not yield any structures of altered mobility on EMSA nor does it produce a DMS footprint. Extension of the quadruplex forming sequence to include a fifth run of guanines (ODN IV) resulted in increased number of altered mobility structures on EMSA and a shift to a broadened maxima positive peak from 260 to 280 nm, together suggesting the formation of a mixture of intermolecular and intramolecular structures with both parallel and antiparallel strand orientations. ODN VI represents an extended HIF-1α PPT (ODN II plus ODN V). ODNs IV and VI both form a core DMS footprint similar to the one seen with ODNs II and III in Figure 4. ODN VII represents a highly homologous sequence in the HIF-2α promoter and differs from the HIF-1α promoter (ODN II) by only 2 nt in the most upstream run of guanines in the quadruplex forming region. This small difference results in four runs of four contiguous guanines, and ODN VII produces a potassium-dependent DMS footprint consistent with the formation of four tetrad stacks as well a CD spectrum in potassium similar to that of ODN II. In summary, these data demonstrate that the sequence from −85 to −65 in the HIF-1α promoter is able to form a parallel, intramolecular G-quadruplex composed of three stacks of tetrads.

DNA polymerase arrest assays have been used as tools for the evaluation of DNA secondary structure formation based on the principle that the polymerase cannot efficiently traverse a stable DNA secondary structure, such as a G-quadruplex, and these assays have proven useful for screening for potential G-quadruplex interactive compounds capable of binding to a given sequence (*70*, *71*). In these assays, we annealed a primer (polP, Table 1) to a template containing either the HIF-1α quadruplex forming region (polH) or a random sequence control (polC), and then

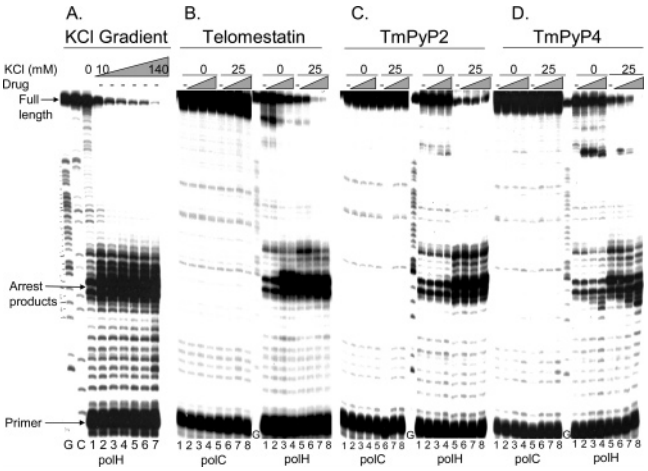


FIGURE 7: DNA polymerase arrest assays show that telomestatin and TmPyP4 can bind to and stabilize the G-quadruplex formed by the HIF-1α promoter. The primer, full-length primer extension products, and DNA polymerase arrest products are shown by arrows. The arrest products occur just before the first guanine of the G-quadruplex and are associated with a decrease in full-length primer extension. G and C ladders were obtained by chain termination sequencing. (A) Extension through the HIF-1α template (polH) after incubation in increasing concentrations of potassium (lane 1: 0 mM KCl; lane 2: 10 mM KCl; lane 3: 25 mM KCl; lane 4: 50 mM KCl; lane 5: 75 mM KCl; lane 6: 100 mM KCl; lane 7: 140 mM KCl). (B) The random sequence control (polC) and HIF-1α templates were treated with telomestatin. (C) Templates were treated with TmPyP2. (D) Templates were treated with TmPyP4. Each template was treated in the absence of drug (lanes 1 and 5), 0.1 μM drug (lanes 2 and 6), 0.5 μM drug (lanes 3 and 7), or 1 μM drug (lanes 4 and 8) in the absence (lanes 1−4) or presence of 25 mM KCl (lanes 5−8).

measured the ability of *taq* DNA polymerase to extend through the template sequence in the presence or absence of potassium and several G-quadruplex binding ligands (Figure 7). A DNA polymerase arrest assay was conducted on the PPT of the HIF-1α promoter (polH) over a concentration range of KCl (Figure 7A). This assay shows the potassium-dependent arrest of taq polymerase at the beginning of the PPT and shows that arrest products become prominent in potassium ion concentrations as low as 10 mM. There is a decrease in full-length primer extension as the potassium concentration is raised to intracellular levels (140 mM). At physiological potassium concentrations, polymerase arrest is almost complete, and we selected a lower potassium concentration, 25 mM, for the addition of potential G-quadruplex ligands so that a decrease in full-length extension products would be detectable and provide evidence of drug binding and stabilization of the G-quadruplex structure. For these assays, we also included a random sequence control template (polC) to rule out any effect of the compounds not due to interaction with the HIF-1α G-quadruplex. Telomestatin is a natural product that was shown to bind to the G-quadruplex formed by the telomeric repeat sequences (*63*, *79*−*82*). The addition of telomestatin (0.1−1.0 μM) to the random sequence control template had no effect on polymerase extension in the presence or absence of potassium ions (Figure 7B). In contrast, telomestatin induced DNA polymerase arrest at the HIF-1α G-quadruplex forming sequence in a dose-dependent manner even in the absence of potassium. In the presence of potassium, polymerase arrest was complete at 0.5−1.0 μM concentrations of telomestatin. These data demonstrate that telomestatin can both induce

and stabilize the HIF-1α G-quadruplex. TmPyP4 is a cationic porphyrin that has been shown to bind to the telomeric G-quadruplex and the human c-myc G-quadruplex, while TmPyP2 is a positional isomer of TmPyP4 that has a lower capacity for binding to G-quadruplexes (*58*, *63*, *65*, *83*). TmPyP2 (0.1−1.0 μM) had no effect on DNA polymerase arrest in the control template nor the HIF-1α template (Figure 7C). In contrast, TmPyP4 caused dose-dependent DNA polymerase arrest at the HIF-1α G-quadruplex forming region at concentrations of 0.5−1.0 μM in the presence of potassium ions but had no effect on the random sequence control template at any concentration tested (Figure 7D). These data demonstrate that TmPyP4 but not TmPyP2 is capable of binding to and stabilizing the G-quadruplex formed by the HIF-1α promoter.

DISCUSSION

In summary, we have demonstrated that a polypurine:polypyrimidine tract in the human HIF-1α promoter is needed for constitutive transcription of the HIF-1α gene. We have shown that the polypurine strand from this region from −65 to −85 (upstream of the transcription start site) can fold into an intramolecular G-quadruplex to produce a potassium-dependent DMS footprint and a characteristic circular dichroism spectrum indicative of a parallel arrangement of the four strands involved in tetraplex formation. We have shown that two G-quadruplex ligands, telomestatin and TmPyP4, are capable of binding to and stabilizing the G-quadruplex formed by the HIF-1α polypurine sequence. We have shown that specifically altering the sequence of the polypurine:polypyrimidine tract within the G-quadruplex forming region markedly reduces HIF-1α transcriptional activity, results that are in broad agreement with a previous HIF-1α promoter serial deletion analysis (*24*). It is interesting to note that the HIF-2α promoter contains a nearly identical polypurine/polypyrimidine tract, and while not the focus of these studies, the polypurine strand (ODN VII) also appears capable of forming an intramolecular G-quadruplex. These observations may indicate that there is a level of coordinate transcriptional regulation for the HIF-1α and HIF-2α genes based on a common structural motif in the promoter regions of these two hypoxia inducible genes that drive the cellular response to hypoxia.

The DMS footprint produced by the HIF-1α PPT suggests the formation of three stacked tetrads from a sequence with the general motif: $G_3N_1G_3N_6G_3N_1G_3$. A sequence derived from the c-myc oncogene, myc-1245, bears the same general sequence motif (Figure 4) and was structurally characterized by nuclear magnetic resonance (NMR) (*67*). These studies showed that when the myc sequence was modified by substitution of one run of guanines to reduce the number of competing structures, the resulting $G_3N_1G_3N_6G_3N_1G_3$ motif produced a parallel, propeller-type G-quadruplex composed of three stacks of G-tetrads and three double-chain-reversal loops. The central loop contained six nucleotides, while the other two loops contained only a single nucleotide. We believe that our data are consistent with a similar structure in the HIF-1α promoter. First, DMS footprints demonstrate that the two runs of three protected guanines at each end of the sequence are separated by guanines in the central loop region that are reactive with DMS. Second, the CD spectra of the HIF-1α G-quadruplex (ODNs II and III) have strong positive peaks at 260 nm, suggesting a parallel orientation of the runs of guanines involved in the formation of the G-tetrads. On the basis of these data, we infer that the HIF-1α promoter can form a parallel, propeller-type G-quadruplex. The presence of additional runs of guanines that could potentially participate in G-quadruplex formation is also similar to the G-quadruplex forming regions in the c-myc and muscle specific gene promoters. In the HIF-1α promoter, a fifth run of three guanines is present just downstream of the G-quadruplex forming region and could participate in the formation of alternate G-quadruplex structures. Indeed, although the region downstream of the −65 to −85 PPT contains multiple runs of guanines, this region (represented by ODN V) does not seem capable of forming a G-quadruplex by itself; however, extended PPTs encompassing −65 to −85 plus downstream sequences (ODN IV and VI) appear to form G-quadruplexes. It is possible that the downstream runs of guanines could form bimolecular G-quadruplexes with the upstream runs of guanines, and precedent for the formation of such complexes has recently been reported in several muscle specific gene promoters (*59*).

G-quaduplex structures in gene promoters could potentially repress or activate gene transcription. For example, a G-quadruplex structure in the human insulin-linked polymorphic region (ILPR) was suggested to enhance the transcription of this gene (*72*). Initial models of the G-quadruplex in the c-Myc promoter proposed an activating role for this structure in transcription (*55*). However, G-quadruplex formation stabilized by TmPyP4 repressed c-myc transcription, suggesting that the G-quadruplex is a transcriptional repressor (*57*). We suggest that the HIF-1α promoter represents another example of a gene that contains an important regulatory element capable of G-quadruplex formation. G-quadruplex formation in this region may be involved in positively or negatively regulating HIF-1α gene expression, and further studies will be needed to determine whether such structures play a role in HIF-1α transcription in the cell. It is important to note that the mutation we used to demonstrate the important role of the polypurine tract in HIF-1α transcription would be expected to abrogate the binding of two activating transcription factors, Sp1 and AP-2, from their putative binding sites, as well as prevent G-quadruplex formation. Further studies will be needed to determine the relative role of transcription factor binding and G-quadruplex formation in the polypurine tract on HIF-1α transcription. A DNA polymerase arrest assay demonstrated that two G-quadruplex ligands, telomestatin and TmPyP4 can bind to and stabilize the G-quadruplex formed by the HIF-1α promoter. Further studies will be needed to determine whether these molecules have an effect on HIF-1α gene expression. Small molecule inhibitors of HIF-1 are currently under investigation as anticancer therapeutics (*84*), and if G-quadruplex ligands were shown to repress HIF-1α expression, such agents could have applications to inhibit tumor angiogenesis and the ability of tumors to adapt to a hypoxic microenvironment.

SUPPORTING INFORMATION AVAILABLE

Extinction coefficients for oligonucleotides. This material is available free of charge via the Internet at http://pubs.acs.org.

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